

Biological/Horticultural Internship Final Report

Shane R. Palmer

KENNEDY SPACE CENTER

Major: Biology

NIFS Internship, Spring Session

Date: 19-04-2017

Biological/Horticultural Internship Final Report

Shane R. Palmer¹ and LaShelle Spencer²
Kennedy Space Center, FL 32899

Nomenclature

<i>GMO</i>	= genetically modified organism
<i>NASA</i>	= National Aeronautics and Space Administration
<i>KSC</i>	= Kennedy Space Center
<i>NIFS</i>	= NASA Interns, Fellows, and Scholars
<i>SLSL</i>	= Space Life Sciences Laboratory
<i>PtFT1</i>	= Flowering locus T1
<i>CO₂</i>	= Carbon dioxide
<i>ppm</i>	= Parts per million. Unit of measuring gas/liquid concentration.
<i>Scion</i>	= Aerial portion of a plant that has been removed and grafted onto another plant
<i>Rootstock</i>	= A rooted plant that the scion is grafted onto.
<i>pH</i>	= Potential of Hydrogen
$\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	= Micromoles per square meter per second. Unit of measuring light intensity.
<i>PAR</i>	= Photosynthetically active radiation.

Abstract

A study was conducted to determine water use requirements of genetically modified (GMO) dwarf plum. GMO plum and unmodified standard plum plants were grown in a controlled environment chamber under varying CO₂ concentrations (400 ppm, 1500 ppm, and 5000 ppm). Pepper plants were also grown in the chamber for additional comparison. Leaf stomatal conductance, biomass accumulation, soil moisture and pot weights were measured; Stomatal conductance of GMO plum and pepper plants decreased at sustained elevated CO₂ concentrations. The stomatal conductance rates of the standard plums, however, increased at sustained elevated CO₂ concentrations. Further data analysis (statistical analysis, biomass, soil moisture and pot weight measurements) is ongoing and required to gain better understanding of the data.

An additional proof-of-concept study was undertaken to determine the feasibility of grafting unmodified standard plum scions onto genetically modified rootstocks as a propagation method. Bud grafts were performed on three GMO plum rootstocks: NASA-5, NASA-10, and NASA-11. All of the standard plum buds grafted onto NASA-5 and NASA-10 rootstocks began growing, indicating that this grafting method is highly successful for the formation of a graft union and initial bud growth. However, bud growth during stem elongation was curtailed on several grafts due to a combination of nutritional deficiency and physical damage/obstruction of the grafted tissues. Bud growth on the NASA-5 rootstock occurred sooner than in grafts on the NASA-10 rootstock, while only one bud graft has shown growth on the NASA-11 rootstock thus far. These marked differences in the onset of bud growth suggest genotypic differences between the rootstocks may affect bud graft vigor. Mature standard plum scions grown on the NASA-5 rootstock appeared to retain most or all of the physical characteristics of the standard plum donor plant.

I. Introduction

Future long duration space flight missions will increasingly rely upon on-site food production systems to supplement crew members' diets. The benefits of such a system are both nutritional (in the form of fresh produce) and psychological (in the form of greater food variety and flavors). GMO dwarf plum varieties are being evaluated as a candidate food crop for such missions. Unlike conventional stone fruit trees, plums (*Prunus domestica*) genetically modified to overexpress the PtFT1 gene exhibit a very compact growth habit, early maturation, and continuous

¹ NIFS Intern, Engineering Services Contract, Kennedy Space Center. Educational Institution: St. Petersburg College

² Scientist and NIFS internship mentor, Engineering Services Contract, Kennedy Space Center.

flowering and fruit production without the need for seasonal chilling and dormancy periods¹. These characteristics make them more suitable for the limited time, space and resources available for growing food onboard spacecraft.

In addition, studies in both animal and human models have shown that plum fruits contain phytonutrients which help prevent and repair bone calcium loss¹⁻³, which is a significant health concern for astronauts due to prolonged microgravity and radiation exposure³. Thus, plum fruits may serve a bioregenerative function for spaceflight missions.

The primary goal of this study was to determine the water use requirements of the genetically-modified plums ('GMO plum') by measuring stomatal conductance rates, soil moisture, pot weights and biomass accumulation at varying CO₂ concentrations. These measurements were compared to those of unmodified 'standard' control plants grown in the same conditions. The results of this study will assist in evaluating the practicality of using GMO plums as food crop plants for future manned space missions.

In addition, a proof-of-concept study was undertaken to determine whether plant tissue from unmodified standard plums could be successfully grafted onto GMO rootstocks, and what, if any, effects the GMO rootstock had upon the unmodified scions.

II. Materials and Methods

A. GMO plum water use study

Modified and unmodified specimens of plum trees (*P. domestica*, 'Blue Byrd' cultivar) were maintained in a controlled environment chamber at the Space Life Sciences Center (SLSL) in Exploration Park, Florida, for the duration of the study. The GMO plums used for this experiment were previously down-selected to a single line (NASA-11). In addition to the plum plants, two bell peppers (*Capsicum annuum*, 'Pompeii' cultivar) were grown in the chamber to compare to the plums.

Lighting in the chamber was provided by a 1:1 ratio of High Pressure Sodium and Metal Halide lamps on a 16hr:8hr light:dark schedule. Plants were fertigated eight times daily with nutrient solution (Peter's granulated nutrient mixture) with an approximate electrical conductivity of 1200-1250 µS and pH 6.00-7.00. Temperature was maintained at approximately 22 degrees Celsius and relative humidity at approximately 50%.

Three varying CO₂ concentrations were used in the chamber; a 'native' CO₂ concentration of 400 ppm (approximate ambient atmospheric conditions), an elevated CO₂ of 1500 ppm, and super-elevated CO₂ at 5000 ppm. CO₂ acclimation occurred in three-week blocks (Table 1), which were then repeated for a total of two replicates for each condition. CO₂ levels were alternated every two days during the first week of each three-week block, and then levels were kept constant during the following two weeks. Morning (approximately 1-2 hours after lights on) and mid-day (approximately 6-8 hours after lights on) leaf porometry readings were taken daily for the first week of each block, and every other day (three times a week) during the two weeks of constant CO₂ concentration.

At the end of each three-week block, all of the plum plants were pruned back to a standard size and the fresh and dry weights of the pruned material were recorded to determine the water content of the plum tissues.

Table 1. CO₂ acclimation testing schematic

Test 1a: 1500 to 400ppm CO ₂ (1/30/17 to 2/16/17)									
Native CO ₂ 1500ppm 2 wk Duration	Day 1		Day 2	Day 3		Day 4	5		Native CO ₂ 400ppm 2 wk Duration
	Stomatal Conductance Msmts	Change CO ₂ to 400ppm	Acclimation Day	Stomatal Conductance Msmts	Change CO ₂ to 1500ppm	Acclimation Day	Stomatal Conductance Msmts	Change CO ₂ to 400ppm	
Test 2a: 400 to 1500ppm CO ₂ (2/20/17 to 3/9/17)									
Native CO ₂ 400ppm 2 wk Duration	Day 1		Day 2	Day 3		Day 4	5		Native CO ₂ 1500ppm 2 wk Duration
	Stomatal Conductance Msmts	Change CO ₂ to 1500ppm	Acclimation Day	Stomatal Conductance Msmts	Change CO ₂ to 400ppm	Acclimation Day	Stomatal Conductance Msmts	Change CO ₂ to 1500ppm	
Test 3a: 1500 to 5000ppm CO ₂ (3/13/17 to 3/30/17)									
Native CO ₂ 1500ppm 2 wk Duration	Day 1		Day 2	Day 3		Day 4	5		Native CO ₂ 5000ppm 2 wk Duration
	Stomatal Conductance Msmts	Change CO ₂ to 5000ppm	Acclimation Day	Stomatal Conductance Msmts	Change CO ₂ to 1500ppm	Acclimation Day	Stomatal Conductance Msmts	Change CO ₂ to 5000ppm	

B. GMO plum grafting experiment

Method 1: Scions from unmodified standard plums were grafted onto three GMO plum rootstocks (one plant each from NASA-5, NASA-10, and NASA-11 strains) using the ‘bud grafting’ method for woody trees as described in the book *Plant Propagation*⁷. Young stems (<1 year old) containing dormant axillary buds were pruned off of standard plum plants to use for scions. If not used immediately, the cut stems were placed in a container of water and covered with plastic to prevent desiccation. When ready to use, leaves were trimmed off of the stem, leaving the petioles still attached to the stem to serve as ‘handles’ for manipulating the excised scion tissue later. A razorblade was used to make a shallow cut about 1.5-2 cm above a dormant bud, then slid downward to excise a portion of the stem and underlying cambium layer along with the bud. The cut was completed about 1.5-2 cm below the bud.

A young stem of similar diameter to the scion was selected on the GMO plum rootstock, and one of its axillary buds was sliced off to create a wound of similar size to the previously excised standard plum scion. The scion bud was lifted by its petiole and positioned onto the wound on the stem of the rootstock, so that their exposed cambium layers were aligned. If necessary, additional cuts to the scion and/or rootstock were made to trim the tissue so that they matched up and were flush with one another. The tissue pieces were gently pressed together while the scion petiole was trimmed off and parafilm grafting tape was wrapped around the entire graft to hold it in place and prevent desiccation. The tape was wrapped from below the start of the graft, upwards over the entire graft, and ended above the graft (Figure 1). In addition, a portion of the grafts were covered with zipper-seal plastic bags to increase humidity and further prevent desiccation. The plastic bags were removed when bud growth was noted.



Figure 1. Completed bud graft that has been wrapped in parafilm tape and labeled. The scion material is from unmodified standard plum and the rootstock is NASA-5 GMO rootstock. Image credit: Shane Palmer, NIFS intern.

The grafting tape was left on the stems for at least three weeks to allow an adequate graft union to form between the scion and rootstock tissues. The grafting tape was carefully removed by cutting it away from the stem with a razorblade. The stems of the rootstock were then pruned back to just above the graft to encourage stem growth on the graft.

All three of the GMO rootstock plants were grown indoors at ambient temperature (approximately 22 degrees Celsius) and relative humidity (approximately 40-50%). Full spectrum lighting was provided by overhead Agrobrite fluorescent light fixtures on a 16hr:8hr light:dark cycle. NASA-5 and NASA-10 rootstocks were grown side by side under a single light fixture with four fluorescent tubes. Grafts on the NASA-5 rootstock were located 31.75cm-48.25cm below the light fixture. Grafts on the NASA-10 rootstock were located 29.2cm-42cm below the light fixture. Light exposure levels at the graft sites on NASA-5 and NASA-10 ranged from $88.56 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ to $179.48 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of PAR.

Due to space limitations, the NASA-11 rootstock was grown on a different light rack under two Agrobrite fixtures (with a total of eight fluorescent tubes). Grafts on the NASA-10 rootstock were located 43cm-71cm below the light fixture. Light exposure levels at the graft sites on NASA-11 ranged from $33.58 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (which was shaded by overhead leaves) to $215.5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of PAR.

When bud growth occurred, stems of the grafted scions were photographed and their length measured to determine growth rate (stem length divided by days after first sign of bud growth). Leaf chlorophyll content of the grafted scions was measured in triplicate on mature leaves (if present) using a Konica Minolta SPAD-502 chlorophyll meter (data not reported here). Leaves of non-grafted stems which were similar in age and size as the grafted scions on the GMO rootstock plants was also measured. The size and shape of the leaves of both grafted scions and non-grafted stems on the rootstock were noted for morphological comparison.

Method 2: As an alternate to single bud grafts, several grafts were attempted using entire stem portions of standard plums which contained multiple leaves and buds. Two plants of the NASA-5 line and two plants of the NASA-10 line were used as rootstocks. Plants were maintained in an environmental growth chamber in the Operations and Checkout building on site at the Kennedy Space Center. Lighting, fertigation, temperature, and humidity were maintained at similar levels to the plants used in Method 1 above.

The bottom end of the scion stem was cut at an angle to expose as much of the cambium layer as possible, and a similar cut was made to the top end of the rootstock stem. Similar to Method 1 above, the stems were aligned and pressed together, and grafting tape wrapped around the union. Unlike in the bud grafting method above, plastic bags were not used as additional protection over the grafts.

III. Results

A. GMO plum water use study

Table 2. Mid-Day average stomatal conductance of unmodified (standard) plum, modified (PtFT1) plum, and pepper plants at 400 ppm and 1500 ppm CO₂ concentrations.

CO ₂	Standard Plum	PtFT1	Pepper
400	494.8	549.6	787.8
1500	523	486.7	431

Three study blocks have been successfully completed (Table 1) at the time of this report, with further data collection ongoing. Mid-day stomatal conductance data for Test 1a (1500ppm → 400ppm CO₂) and Test 2a (400ppm → 1500ppm CO₂) are reported in Table 2, Figure 1 and Figure 2. Initial results showed that during Test 1a (Figure. 1), stomatal conductance at varying CO₂ concentrations was highest in pepper throughout the majority of the testing period. It is important to note the presence of intumescence on pepper leave tissue during the entirety of testing.

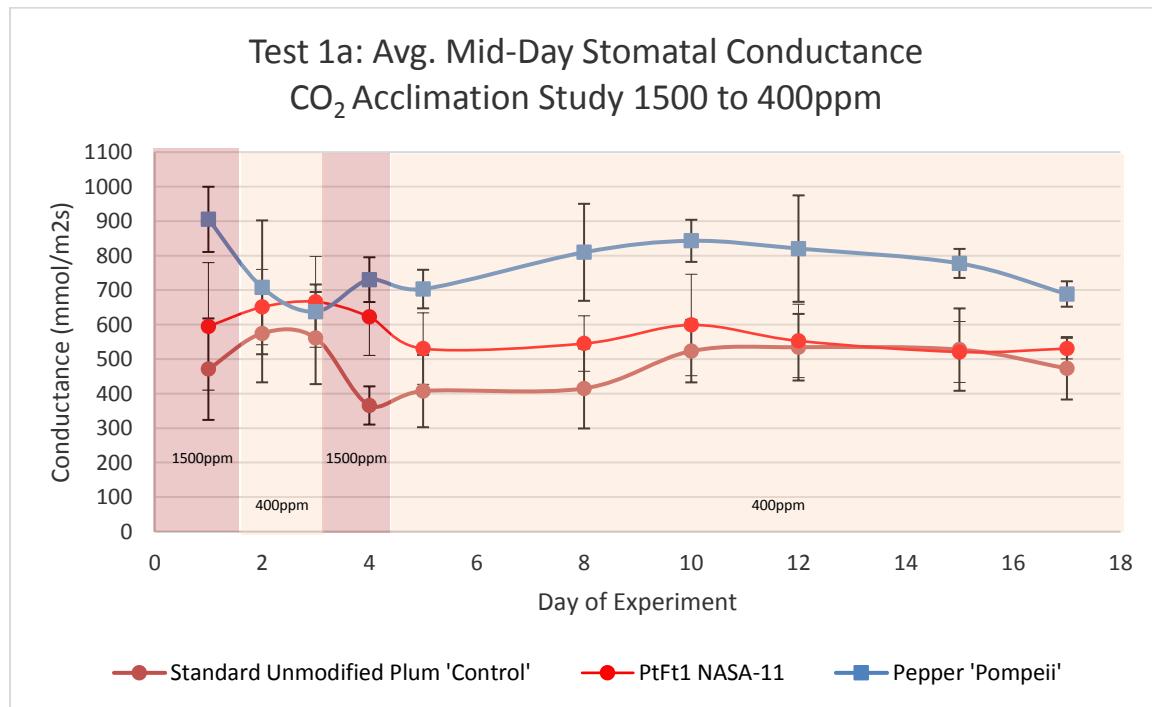


Figure 2. Test 1a; Stomatal conductance of standard plum, PtFT1 Plum and pepper (cv. 'Pompeii') exposed to varying CO₂ concentrations.

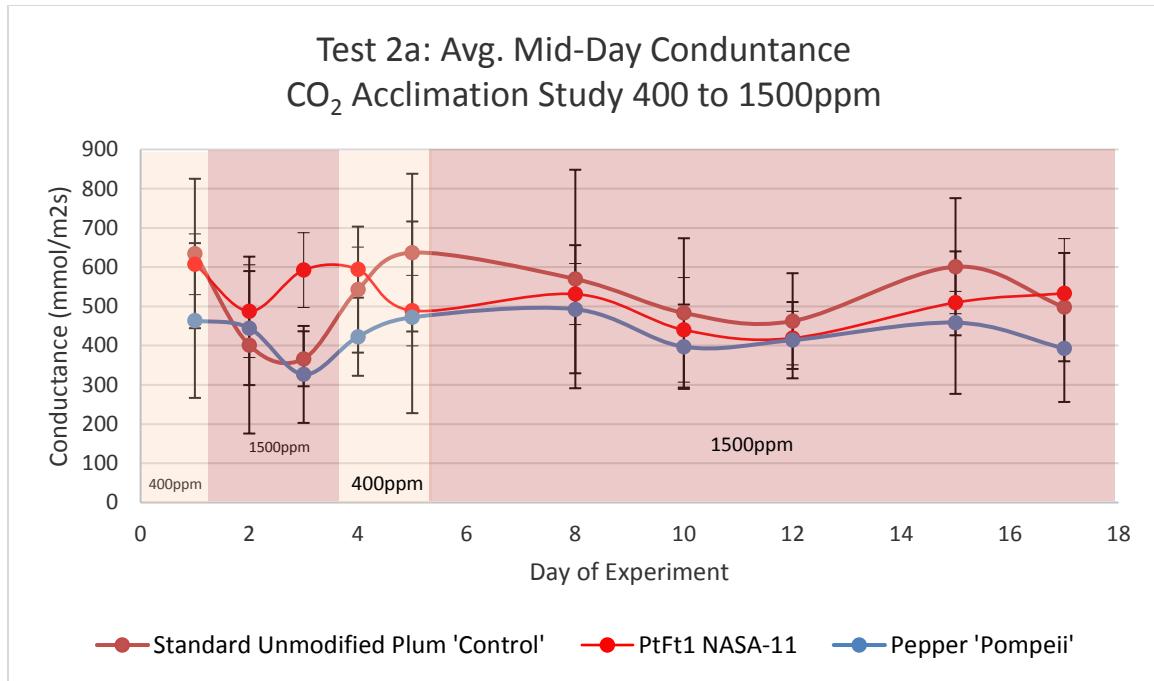


Figure 3. Test 2a; Stomatal conductance of standard plum, PtFt1 Plum and pepper (cv. 'Pompeii') exposed to varying CO₂ concentrations.

Average mid-day stomatal conductance rates of GMO plums were lower during the two-week period of elevated (1500ppm) CO₂ concentrations than during the two-week period of 'native' (400ppm) CO₂ concentration (Figure 1 and Figure 2). Similarly, the pepper plants also exhibited lower stomatal conductance rates during elevated CO₂ concentrations. However, the average stomatal conductance rates of the standard plums were higher during sustained elevated CO₂ concentration than during native CO₂ concentration. Under short-term (2 days) exposure to elevated CO₂ concentration, the stomatal conductance of the standard plum plants was lower than during the short-term exposure to native CO₂ concentration.

B. GMO plum grafting experiment

Method 1: Seven bud grafts were performed on the NASA-5 GMO rootstock, five were performed on the NASA-10 GMO rootstock, and six were performed on the NASA-11 GMO rootstock. All seven buds on the NASA-5 rootstock showed signs of growth after four weeks, with one bud beginning to grow only six days after grafting. One of the grafted buds was damaged during grafting and the graft union did not completely fuse. Despite this, the bud still showed signs of growth and a bulbous callus formed over the exposed graft union (Figure 4).

All five bud grafts on the NASA-10 rootstock remained dormant until after the rootstock's stems were pruned back; they began growing approximately 1 week after pruning (Figure 5). One of the five buds stopped growing and turned brown shortly afterward. As of the writing of this report, only one bud out of the six grafted buds on the NASA-11 rootstock has showed signs of initial growth; four have remained dormant, even after the rootstock stems were pruned back to encourage bud growth. One bud, which was not fully dormant at the time of grafting, turned brown and fell off of the graft about two weeks after grafting.



Figure 4. Imperfect graft union of standard scion grafted onto NASA-5 rootstock, showing callus growth and an oddly persistent bud. Image credit: Shane Palmer, NIFS intern.

About two to three weeks after initial bud growth occurred on the scions grafted to the NASA-5 rootstock, the young leaf tips on every grafted bud developed a brown necrosis around their edges (Figure 6). The growing tips of non-grafted buds on the same rootstock also showed signs of this tip burn. Since the buds at this stage were not close enough to the light source to be experiencing thermal damage, the cause of the necrosis was assumed to be nutritional deficiency and Peter's Liquid S.T.E.M was added to the nutrient solution to add more micronutrients. As of the writing of this report, three of the stems had recovered and returned to normal vigorous growth, while growth was retarded on the remaining buds/stems, likely due to damaged meristem tissue.



Figure 5. Left: First day of visible bud growth on standard scion grafted onto NASA-10 rootstock, roughly one week after pruning back the rootstock to force bud growth. Note healed graft union around the bud. Right: The same bud photographed four days later. Image credit: Shane Palmer, NIFS intern.



Figure 6. Stem of standard plum scion grafted onto NASA-5 rootstock, showing tip burn on young leaves. Image credit: Shane Palmer, NIFS intern.

Mature leaves from standard plum scions grafted onto the NASA-5 rootstock exhibited similar size and appearance (shape, texture, etc.) as the non-grafted standard plum donor plant. The scion leaves were physically dissimilar to the non-grafted leaves of the NASA-5 rootstock, and despite their young age, had already matched or exceeded the size of the largest (and older) non-grafted leaves on the rootstock (Figure 7).



Figure 7. Left: Leaf of standard plum scion grafted onto NASA-5 rootstock. **Right:** non-grafted leaf from the same NASA-5 rootstock. Image credit: Shane Palmer, NIFS intern.

The base of the scion stems where the graft union interfaced with the rootstock was swollen and had grown thicker than the rootstock stem that supported it (Figure 8).

Method 2: All of the stem grafts that were attempted showed signs of water loss and wilt within an hour of grafting, and were dead in less than a week (Figure 9).



Figure 8. Graft union of standard plum scion on NASA-5 rootstock, showing swollen base and thick stem of the scion supported by the smaller stem of the rootstock. Image credit: Shane Palmer, NIFS intern.



Figure 9. Stem of standard plum scion beginning to wilt less than one hour after attempted stem graft onto NASA-5 rootstock. Image credit: Shane Palmer, NIFS intern.

IV. Discussion

A. GMO plum water use study

The porometry results for the GMO plum and pepper plants were consistent with the hypothesis that stomatal conductance would decrease at sustained elevated CO₂ concentrations. The results for the standard plum, however, were not consistent with this hypothesis. Instead, the stomatal conductance rates of the standard plums increased at sustained elevated CO₂ concentrations. This difference was only slightly higher, however, and error bars suggest this may not be significant. Further statistical analysis is required to gain better understanding of the data.

Observations of GMO plums in cultivation at the SSSL suggests they may lose water and wilt more quickly and/or are less drought tolerant than unmodified control plants. Overexpression of the *Flowering Locus T* gene in the guard cells of leaf stomata is known to increase transpiration from leaf tissues⁵, so it is likely that this mechanism is contributing to the observed behaviors in the GMO plum.

B. GMO plum grafting experiment

All of the unmodified standard plum buds grafted onto the NASA-5 and NASA-10 rootstocks began growing, indicating that this grafting method is highly successful for the formation of a graft union and initial bud growth. The grafted buds seemed to exhibit two distinct growth phases; a period of initial bud expansion and development of the first whorl of leaves, and then a period of stem elongation and development of additional ‘primary’ leaves along the stem. Initiation of the secondary growth phase was inconsistent between grafts, and several buds on both NASA-5 and NASA-10 rootstocks stopped growing. Additional external factors such as physical damage/obstruction of the bud and nutritional deficiency likely hindered this long term growth of several grafted buds on the NASA-5 plant, however the three actively growing buds that are present are large and vigorous. While initiation of bud growth was retarded in the NASA-10 rootstock, four of the five grafts did begin growing shortly after the rootstock stems were pruned back, and two of those grafts are now actively growing and approaching a similar size as the three large grafts on NASA-5. NASA-10 has been previously observed to have slower, less vigorous growth than NASA-5, so pruning may be more necessary to ‘force’ bud growth in the grafts when using this line as a rootstock. Grafts on the NASA-11 rootstock were performed later than the prior two rootstocks, and thus far only one bud has shown initial growth (and has since stopped growing). These marked differences in the onset of bud growth suggest that genotypic differences between the rootstocks may affect bud graft vigor.

The failure of all of the attempted stem grafts was likely due to wilt/excessive water loss. While stem grafts on temperate fruit trees are typically performed on dormant plants⁶, the stem grafts attempted in this study used tender, actively growing shoots for scion material with intact leaves that began to droop and wilt from water loss within an hour of grafting. Since the plants grown in the controlled environment never experience winter conditions that would induce dormancy, future attempts at stem grafting should use scions whose leaves have been trimmed or removed to reduce transpiration. In addition, placing plastic bags or some other water-impermeable covering over the entire graft assembly during the first several weeks of healing may further help reduce water loss in the scion and improve the chances of a successful graft union.

The parafilm grafting tape used to wrap the fresh grafts was somewhat difficult to remove. Special care had to be taken not to injure the underlying stem or scion bud with the razorblade when cutting the tape away, and the tape had to be carefully pulled back from the stem so as not to pull off tender leaves or bud tissue with it. In addition, it was learned that the most effective way to wrap the tape was from the bottom up, and to wrap the tape over the bud in a way that the edge of the tape fell just above the bud, so that as the bud grew it could push out through the gap in the tape.

Mature stems from standard plum scions grown on the NASA-5 rootstock appeared to retain most or all of the physical characteristics of the standard plum parent plant. Since the leaves of the scions are still relatively young, they will likely continue to grow in size as they age in a similar manner as their standard plum parent plant, and will greatly exceed the size of the non-grafted leaves on the NASA-5 rootstock. Additional data (porometry, stem structure and growth habit, onset of flowering, etc.) can be taken as the scions age and mature to determine whether there were any biological changes induced by interactions with the GMO rootstocks, or whether the scions retain all of the characteristics of the standard plum donor plants.

While leaf chlorophyll content of the non-grafted standard plum plant was measured (not reported in this document), the comparison of that data to the leaf chlorophyll content of the grafted scions and non-grafted stems on the GMO rootstock is tenuous because the standard plum plant was growing on a different rack with different lighting conditions (partially shaded, and further from the light source) than the rootstock plants. More appropriate statistical comparisons could be made if these external conditions were controlled, however the situation could not be avoided due to space limitations in the lab. Also, better statistical comparisons could be made if self-grafts of GMO-on-GMO and standard-on-standard plum plants were also tested in order to rule out effects caused by the physical act of grafting rather than the biological interactions between scion and rootstock. Additional comparisons between grafted scions, non-grafted GMO rootstocks, and standard plums could be made using porometry to measure leaf stomatal conductance rates.

Acknowledgments

The author would like to thank LaShelle Spencer for her mentorship throughout this project, and for producing the tables and figures and initial data analysis for the water use study. Additional thanks go to the members of the NASA Veggie research group at the Kennedy Space Center for their assistance and input. Funding for the Biological/Horticultural internship was provided by the NIFS program. The author would also like to thank the faculty and administrators of St. Petersburg College for the academic and professional support and preparation they provided for this opportunity.

References

¹Graham, T., Scorza, R., Wheeler, R., Smith, B.J., Dardick, C., Dixit, A., Raines, D., Callahan, A., Srinivasan, C., Spencer, L., "Over-expression of FT1 in Plum (*Prunus domestica*) Results in Phenotypes Compatible with Spaceflight: A Potential New Candidate Crop for Bioregenerative Life Support Systems." *Gravitational and Space Research*, Vol. 3, No. 1, July 2015, pp. 39-50.

² Schreurs, A.S., Shirazi-Fard, Y., Shahnazari, M., Alwood, J.S., Truong, T.A., Tahimic, C.G.T., Limoli, C.L., Turner, N.D., Halloran, B. and Globus, R.K., "Dried Plum Diet Protects from Bone Loss Caused by Ionizing Radiation." *Scientific reports*, Vol. 6: 21343, 11 Feb. 2016.

³ Smith, B.J., Bu, S.Y., Wang, Y., Rendina, E., Lim, Y.F., Marlow, D., Clarke, S.L., Cullen, D.M. and Lucas, E.A., "A Comparative Study of The Bone Metabolic Response to Dried Plum Supplementation and PTH Treatment in Adult, Osteopenic Ovariectomized Rat." *Bone*, Vol. 58, 2014, pp. 151-159.

⁴ Smith, S.M., Zwart, S.R., Heer, M., Hudson, E.K., Shackelford, L. and Morgan, J.L., "Men and Women in Space: Bone Loss and Kidney Stone Risk after Long-duration Spaceflight." *Journal of Bone and Mineral Research*, Vol. 29, No. 7, 2014, pp.1639-45.

⁵ Kinoshita, T., Ono, N., Hayashi, Y., Morimoto, S., Nakamura, S., Soda, M., Kato, Y., Ohnishi, M., Nakano, T., Inoue, S.I. and Shimazaki, K.I., "Flowering Locus T Regulates Stomatal Opening." *Current Biology*, Vol. 21, No. 14, 2011, pp.1232-1238.

⁶ Olmstead, M., *Propagating Fruit Plants in Florida*, University of Florida, IFAS Extension, Gainesville, FL, 2011.

⁷Toogood A, *Plant propagation*, American horticulture society, DK Publishing, New York, 1999, pp. 56-63.